CARBOHYDRATE AND NON-CARBOHYDRATE LIGANDS FOR THE C-TYPE LECTIN-LIKE RECEPTORS OF NATURAL KILLER CELLS. A REVIEW

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Dedicated to the 50th anniversary of the foundation of the Department of Biochemistry, the first biochemical department in Czechoslovakia.

The superfamily of C-type animal lectins is defined by a sequence motif of the carbohydrate-recognition domains (CRDs) and comprises seven groups of molecules. The soluble proteins are group I proteoglycans, group III collectins, and group VII containing the isolated CRDs. Type I membrane proteins include group IV selectins and group VI macrophage receptors and related molecules. Type II membrane proteins are group II hepatic lectins and group V natural killer cell receptors. The latter group has recently attracted considerable attention of the biomedical community. These receptors are arranged at the surface of lymphocytes as homo- or heterodimers composed of two polypeptides consisting of N-terminal peptide tails responsible for signaling, transmembrane domain, neck regions of varying length, and C-terminal lectin-like domains (CTLDs). Since this group is evolutionarily most distant from the rest of C-type animal lectins, the sequence of the C-terminal ligand-binding domain has diversified to accommodate other ligands than calcium or carbohydrates. These domains are referred to as natural killer domains (NKDs) forming a large percentage of CTLDs in vertebrates. Here are summarized the data indicating that calcium, carbohydrates, peptides, and large proteins such as major histocompatibility complex (MHC) class I can all be ligands for NKDs. The wide range of ligands that can be recognized by NKDs includes some new, unexpected compounds such as signal peptide-derived fragments, heat shock proteins, or oxi-

dized lipids. The biological importance of this extended range of recognition abilities is also discussed. A review with 134 references.

Keywords: Calcium; Carbohydrates; Carbohydrate-binding protein; Lectins; Oligosaccharides; Ligands; Peptides; Proteins; MHC class I; Heat shock protein; Signal peptide; Processing; Activating and inhibitory receptors; Adaptor proteins.

1. INTRODUCTION

Lectins are proteins or glycoproteins of non-immunoglobulin nature that have the ability to bind carbohydrates without altering their structure. This interesting class of proteins has been first studied in plants where lectins belong to the most toxic components identified so far. Their unique biological activities such as the ability to agglutinate red blood cells or their exquisite toxicity have been the subject of many early studies at the end of the 19th and beginning of the 20th century[1]. However, in the last century it took another 50 years before the nature of the interaction of these proteins with their carbohydrate ligands was discovered[1]. In the early 1970's, first observations about lectins and their physiological functions in vertebrates were made. It has been since realized that lectin–carbohydrate interactions are involved in several immune recognition processes such as pathogen recognition and neutralization, interactions between leukocytes and the inflammatory epithelium, lymphocyte trafficking and natural killing of various cellular targets[2]. For the latter function, natural killer cells evolved a variety of cell surface receptors belonging to both immunoglobulin (killer cell receptor, KIR)[+] and C-type lectin families[3]. This review concentrates on the most widespread family of animal lectins, namely on the calcium-dependent (C-type) lectins. Diversification of this protein family will be followed from molecules involved in binding of calcium and carbohydrate into a more universal group of proteins. Special emphasis will be placed on critical evaluation of the data indicating how the binding surface of these proteins became reorganized to accommodate a variety of ligands including calcium, carbohydrates, proteins, peptides, oxidized lipids and other structural classes of ligands.

+ *Abbreviations used*: CRD, carbohydrate-recognition domain; CTLD, C-type lectin-like domain; IgE, immunoglobulin E; ITIM, immunoreceptor tyrosin inhibitory motif; KIR, killer cell receptor; MBP, mannose binding protein; MHC, major histocompatibility complex; NK, natural killer; NKD, natural killer cell domain.

2. EVOLUTIONARY CONSIDERATIONS

The common sequence motif defining the carbohydrate-recognition domain (CRD) of C-type lectins has been widely used to identify structurally related modules in other proteins in a wide range of model organisms for which sufficient sequence data became available. In the earthworm *Caennorhabditis elegans*, protein modules related to the C-type carbohydrate-recognition domains of animal lectins was found in at least 125 proteins containing a total of 183 C-type lectin-like domains[4]. However, only 19 of these domains showed conservation of the amino acids ligating calcium, and only 7 displayed sequence similar to domains with the documented ability to bind mannose and *N*-acetylglucosamine[4]. In the fruit fly *Drosophila melanogaster*, it has been found that C-type lectin-like domains may play roles distinct from those in vertebrates[5]. These differing functions have also been recently documented in *Hydra vulgaris*, which produces a novel type of receptor named "sweet tooth" containing protein tyrosine kinase domains connected with extracellular C-type lectin-like domains[6].

One of the largest group of vertebrate proteins identified by computer analysis of the sequence data from mouse, rat and human genomes consists of type II membrane proteins encoded in the natural killer (NK) gene complex[7]. In these receptors, the C-terminal CRD-like sequences are linked by neck regions of varying length to signal-anchor sequences and N-terminal cytoplasmic tails involved in signaling through these molecules. Depending on the peptide motifs present in the cytoplasmic tails, these molecules may associate with protein tyrosine kinases[8], protein phosphatases[9], or with various adaptor proteins[10], and thus deliver either inhibitory or activating signals to natural killer cells on the surface of which they are expressed. These receptors thus participate, together with the type I membrane receptors of the immunoglobulin family[11], in a wide range of signaling events regulating the effector functions of killer lymphocytes. One subgroup of these proteins represented by Ly49 in rodents mediates inactivation of NK cells when they are engaged by class I major histocompatibility antigens on the potential target cells[12] (Fig. 1). This interaction serves to inhibit killing of most normal cells of the body. The second group of receptors represented by NKR-P1 protein can activate killing of those cells that express low surface amounts of major histocompatibility complex (MHC) class I, and in which the dominant inhibitory cascade shown in Fig. 1 thus may not apply.

The recent whole-scale sequencing of human and rodent genomes revealed many additional receptors involved in either activating or inhibitory

pathways. Interestingly, the genes for all of these receptors are concentrated in the ca. 300 kbp region of human chromosome 12 (and the corresponding mouse chromosome 6 and rat chromosome 4) named the NK gene complex[13,14] (Fig. 2). The genomic data have now provided a basis for a definitive knowledge of the overall number and arrangement of the NK cell lectin-like receptors. When we compare the NK gene complex in humans and in rodents (mice and rats), many orthologous molecules are evident, although not all the ortholog triads have been identified yet[15,16]. Humans seem to have more activating receptors for which no obvious relatives have been identified in mice. On the other hand, the large mouse family of Ly49 antigens has only a very limited mirror in humans (see Fig. 2) in the form of Ly49L pseudogene for which mature mRNA transcripts or receptor proteins could not be identified[3]. The presence in the putative CRDs of

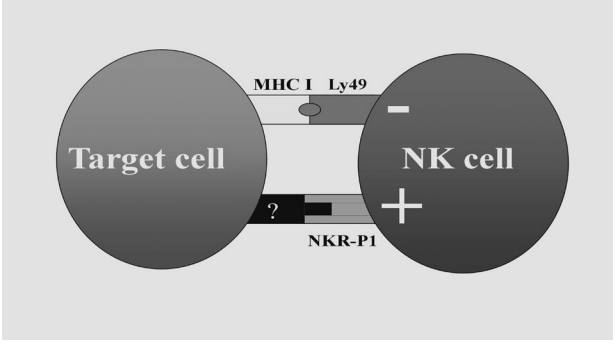

FIG. 1
The role of activation (+) and inhibitory (–) receptors in natural killing

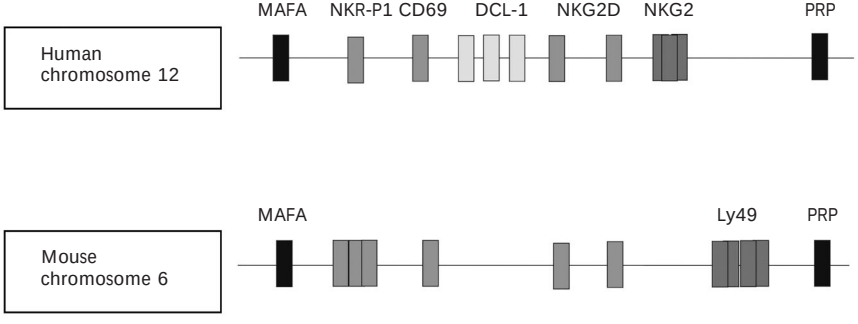

FIG. 2
Scheme of the genes within the NK cell complex

these receptors of many residues common to the classic animal C-type lectins[17] initially led to the suggestion that the C-terminal portion of NK cell receptors may be related to animal lectins in both structure and function. However, careful examination of the sequence data for these molecules indicates that such an interpretation may be only partially corect, and similarities in both structure and function must be verified experimentally for each of the individual receptor under consideration. Despite the limited number of NK cell receptors, this is still a formidable task considering how complex and controversial these evaluations might be for each individual receptor (cf. examples provided in the following sections). Computer methods such as multiple sequence alignment and cluster analysis clearly demonstrate that the ligand-binding domains of NK cell receptors are evolutionarily quite divergent from the classic C-type lectins, and form a distinct evolutionary lineage[17]. The evolutionary tree shown in Fig. 3 and the published multiple sequence alignment[2] reveal both large variations within the NK cell receptor family, and the fact that residues characteristic of the C-type lectin CRDs are only partially conserved in the NK cell receptors. Most obvious is the conservation in the CRD of the two pairs of cysteines that form the two disulfide bonds (the outer and the inner). Many of the remaining residues of the C-type CRD motif are aromatic and aliphatic hydrophobic amino acids, which form the two hydrophobic cores of the domain (lower and upper core[18]) in the form of two α helices and 3 pairs of antiparallel β-sheets. On this conserved structural framework, individual

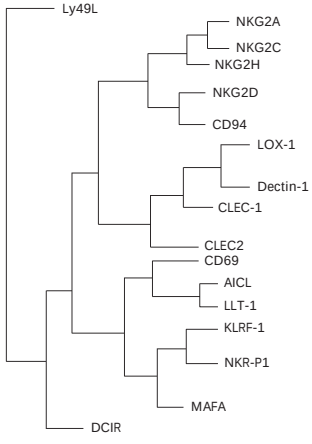

FIG. 3
Evolutionary tree of the NK cell receptor belonging to C-type lectin family

ligand-binding surfaces are modeled very efficiently mainly through two variable loops (3 and 4) in the membrane-distal part of the molecule (Fig. 4). The remaining residues characteristic of CRDs in classic C-type lectins are those ligating calcium 1 and calcium 2, many of which are also involved in binding of carbohydrates. Importantly, these amino acid residues are almost completely absent in the NK cell receptors. The few hydrophobic residues in classic C-type lectins that are not conserved in the NK cell receptors are clustered near the Ca^{2+} ligating amino acids at the sugar-binding end of the domain. Particularly interesting is the lack of conservation of the tryptophan and proline residues in loops 3 and 4. In mannose binding protein (MBP), which is a prototype example of the classic C-type animal lectins, the proline 186 is packed against the tryptophan side chain, and assumes a *cis* configuration, thus establishing the conformation of these two loops and positioning the adjacent Ca^{2+} ligating residues to form the carbohydrate-binding site[18,19]. A highly conserved tryptophan 204 in the β-strand 4 forms a part of the core of MBP and interacts at right angles with another tryptophan in loop 3. The combined absence of the residues discussed above suggests that the conformation of the ligand-binding membrane-distal part in NK receptors differs substantially from the classic C-type lectins. The lack of conservation of both the polar Ca^{2+} ligating residues and the hydrophobic residues in loops 3 and 4 suggests that the Ca^{2+} binding sites in the NK cell receptors may be in positions completely different from those in classic C-type lectins. Finally, the membrane-distal ligand-binding portion in NK cell receptors has been substantially remod-

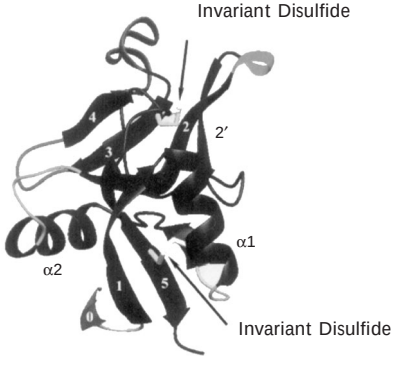

FIG. 4
Structure of the CTLD with elements of structural framework (dark color), and two variable ligand-binding loops (3 and 4) indicated in the upper part. Helix α2 is not conserved in some NK cell receptors such as CD94 [55]

elled to accommodate additional classes of ligands other then calcium and carbohydrates, which are the ligands for classic C-type lectins. Since the designation of the C-terminal domain of NK cell receptors as CRD may be misleading from the point of view of lack of carbohydrate binding, it has been suggested to introduce a larger family of protein modules designated as C-type lectin-like domains[2] (CTLD). According to this nomenclature, the C-terminal domains of NK cell receptors are named NK cell domains (NKD), and they represent a subfamily of CTLDs that are relatively structurally divergent from CRDs of the classic C-type lectins to accommodate a large structural variability of the potential ligands (Fig. 5).

3. BINDING OF CALCIUM BY NKD MODULES

For experimental evaluations of the ligand-binding activities of NK cell receptors, suitable molecular probes with the defined arrangement of the C-terminal ligand-binding modules should be used. Since these receptors are expressed at the surface of NK cells as homo- or heterodimers (Fig. 6), the use of the dimeric soluble proteins corresponding to the entire extracellular portion of the molecule would represent a natural choice. However, one should keep in mind that interactions measured with such soluble proteins in vitro may still be far away from the physiological interactions on the surface of NK cells. Membrane-bound receptors can behave differently from the soluble receptors depending on their particular protein architecture and details of the interchain disulfide bonding[20]. The plasmatic membrane itself may influence the binding conformation of the receptor, as can other, receptor-associate membrane proteins. Moreover, we must assume the clustering of the receptor both upon binding of the

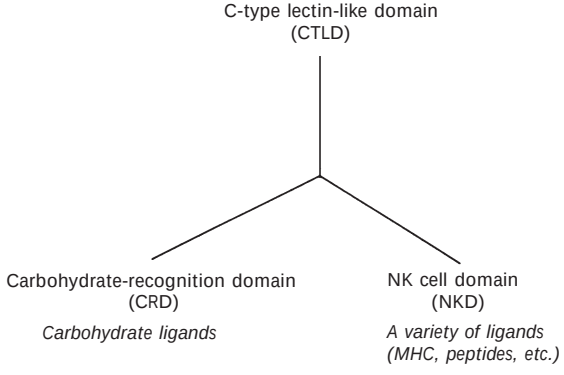

FIG. 5
Designation of the ligand-binding domains within C-type lectin family[2]

high-affinity ligands (outside – in-signaling) and upon receiving the intracellular signals from other receptors (inside – out-signaling). Even with all the limitations mentioned, the preparation of well folded dimeric proteins e.g. by recombinant prokaryotic or eukaryotic expression is often very difficult, and only several examples of the successful use of alternative probes have been published. The monomeric receptors corresponding to the NKD domains are small (approximately 100 amino acids) proteins, which may, due to their size, bring an advantage for biochemical and structural studies, such as heteronuclear NMR experiments. The major limitation for the use of these otherwise convenient probes is often the low affinity of the binding to ligands, in particular complex ligands, for which the oligomeric proteins may have higher affinity. Because of this disadvantage, which is an important problem in ligand identification studies, proteins containing oligomeric (e.g. tetrameric) NKDs have been prepared. The oligomerization greatly facilitates successful identification of ligands, but is also prone to various artefacts coming from the non-physiological arrangement of NKDs. On the other hand, the use of proteins with highly clustered domains or soluble aggregates in binding studies should be avoided[21].

Although the presence or absence of calcium in the ligand-binding site of NK cell receptors is often estimated from the modulation of binding by the addition of calcium or chelators, there are obvious problems with this type of approach, and very few rigorous studies have been published so far. For instance, there is evidence that the mouse inhibitory receptor Ly49 binds to certain anionic polysaccharides in Ca^{2+} independent fashion[22]. This finding would be consistent with the suggestion that carbohydrate-binding site(s) in Ly49A would be quite different from those in classic C-type lectins. On the other hand, such sites have already been detected in selectins, where

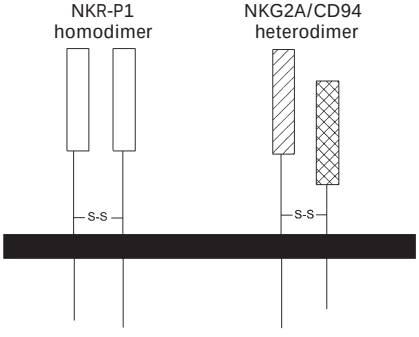

FIG. 6
Homodimeric and heterodimeric arrangement of NK cell lectin-like receptors

they are responsible for an interaction of these cell adhesion molecules with the sulfatides[23]. Biochemical analyses performed in our laboratory established the details of calcium binding to rat NKR-P1A and human CD69, two important activating NK cell receptors. These studies not only revealed the important features that are unique for the binding of calcium by each of these receptors, but also pointed to the difficulties in assessing the "calcium-dependent" ligand binding using indirect approaches. In the case of NKR-P1A receptor, the initial biochemical study revealed that calcium was essential for the correct folding of this protein in vitro. Calcium was found to form an integral part of the NKD of this receptor in the form of one mol of calcium bound per mol of the domain[24]. However, under neutral (physiological) pH, the calcium ion remained strongly bound to the protein and could not be removed by chelating agents such as EDTA [24]. The calcium could be released from the domain in both acid and alkaline environment, once again pointing to principal differences between the NK receptors and classic C-type lectins in which the binding of calcium remained stable in the alkaline environment. Additional structural studies revealed further details of calcium binding in the NKR-P1A receptor[25]. First, two molecular models of NKR-P1A domains have been constructed, and candidate calcium-ligating amino acids identified as Asp^{74} in Model I and Glu^{100} in Model II (Fig. 7). In order to distinguish between the two models, protein in which the two calcium-ligating amino acids have been replaced by alanine has been produced, and binding of calcium to these proteins was determined experimentally. The binding of calcium measured by direct binding assay using $^{45}Ca^{2+}$ was abrogated only when the Asp^{74} was mutated, while in the Glu^{100} mutant the binding was identical to the wild-type

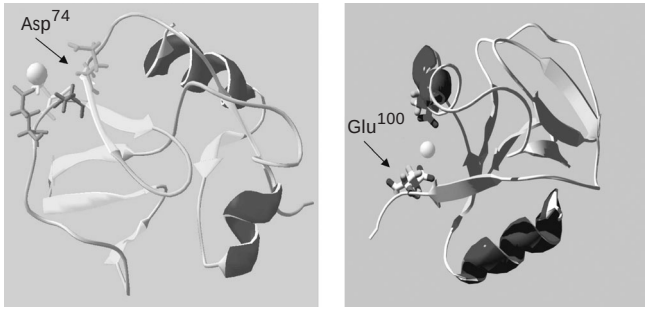

FIG. 7
Molecular models of calcium-binding sites in NK cell receptor NKR-P1. The key amino acids used in experimental verifications of the models are indicated. Model I (on the left) proved correct by this verification

protein. Detailed examination of the calcium-binding site suggested by Model I revealed the presence of two calcium-ligating serines (Fig. 7). Their presence would explain the pH dependence mentioned above. Finally, the role of calcium in NKR-P1 is to support the overall structure of this protein fold, but no direct role in binding of other NKR-P1 ligands such as GlcNAc has been found[25].

The second protein that has been subjected to detailed examination with regard to Ca^{2+} binding is the early activation antigen of lymphocytes CD69. The reason for an examination of the details of calcium binding in this particular protein was motivated by certain controversial observations made with respect to the recognition of carbohydrates by this receptor[26,27]. Although our initial study established calcium-dependent binding to certain carbohydrates[26], these finding have later been questioned using an alternative expression construct[27]. The data from molecular modeling and the subsequent structural studies of CD69 by protein crystallography did not shed much light into the lasting controversy[28–30]. In order to address these discrepancies, we have established an alternative recombinant expression system in which the NKD of this receptor was expressed and refolded in the active form as a Ser^{100}-Lys^{199} segment lacking any extra amino acids originating from the expression vector[31]. We checked the correct folding of our protein using several spectral techniques, and examined the conditions of calcium binding. When the NKD from CD69 was fully calcified, and then subjected to dialyses under various conditions, 1 mol of calcium per mol NKD remained bound to the protein under neutral pH. Under acid conditions, all of the calcium was lost between pH 4 and 5. Molecular modeling allowed to identify amino acid residues involved in calcium binding as Asp^{171}, Glu^{185} and Glu^{187}. Direct binding assays that again used $^{45}Ca^{2+}$ and wild-type protein confirmed the existence of a single Ca^{2+} binding site in CD69 with K_d of 54 μmol l^{-1}. Mutation of any of the above three amino acids resulted in significant impairment of calcium binding. The pH dependence of calcium binding in this example followed the usual dependence observed for the classic C-type lectins providing additional evidence that carboxyles of the acid amino acids represent the only calcium-chelating structures[31].

4. BINDING OF CARBOHYDRATES BY NKD MODULES

As already mentioned in the preceding section, calcium-independent binding of the mouse inhibitory NK receptor Ly49 to certain acid polysaccharides has been reported by at least two groups[22,32]. Binding of MHC class I

glycoproteins to inhibitory NK cell receptors is facilitated by N-glycosylation of the ligands. A hypothesis to explain these findings is that a protein–carbohydrate interaction is involved at the initial stage of the interaction to help to "dock" the MHC class I glycoprotein in the binding site of the NK cell receptor after which stronger protein–protein interactions occur. However, these findings still remain controversial since the mutation of N-glycosylation acceptor sites in MHC class I D^d did not reduce inhibitory NK cell recognition in one study[33], and reduced it only partially in another[34].

In our laboratory we have investigated molecular details of carbohydrate binding by the two classic activating receptors of NK cells, rat NKR-P1 and human CD69. With regard to molecular details of NKR-P1–carbohydrate interactions, we have used both monomeric and dimeric prokaryotic recombinant proteins, and evaluated the initial monosaccharide binding specificity. The order of inhibitory potency was ManNAc >> GalNAc > GlcNAc >> Fuc >> Man > Gal [24,35]. While hexosamines obtained by de-*N*-acetylation of the above carbohydrates are very poor ligands, the additional hydrophobic aglycon significantly enhanced the binding, especially in the case of β-*O*-glycosidic linkage[35]. The affinities to several *N*-acetylhexosamine-based oligosaccharides are significantly higher compared to the monosaccharides, peaking at the level of chitotetrose[36]. Introducing systematic alterations into this model ligand, both at the nonreducing and at the reducing end, resulted in a disaccharide GalNAcβ1→4ManNAc and a trisaccharide GalNAcβ1→4GlcNAcβ1→4ManNAc, both of which turned out to be extremely potent neutral saccharide ligands (IC_{50} = 10^{-9} and 10^{-10} mol l^{-1}, respectively).

Molecular modeling and direct binding experiments using the radiolabeled oligosaccharide ligands and both wild-type and mutant NKD of rat NKR-P1 revealed the existence of a binding groove extending over the entire domain. The orientation of this ligand-binding groove would place the nonreducing GalNAc of the above trisaccharide into the membrane-proximal position, while the binding of the reducing ManNAc would occur in the membrane-distal part of the receptor[25]. The binding groove is lined with several amino acids directly involved in the ligation of carbohydrates. Of these amino acids, Val^{177} and Ser^{179} are of a particular interest since they belong to the few amino acids that are altered in rat NKR-P1B, a minor natural variant of the prevalent NKR-P1A isoform having 97% amino acid identities within the NKD residues. Small number of amino acid substitutions between these two isoforms is surprising in view of the oposite function of NKR-P1B, which was shown to have inhibitory rather than activat-

ing function in both the rat and the mouse[37–39]. Interestingly, the critical amino acid residues responsible for this different specificity seem to be placed along the oligosaccharide-binding groove. This groove seems to be blocked by NKR-P1B amino acids in a way that allows the monosaccharide ligands to be bound in unaltered form, but completely prevents the binding of oligosaccharide or complex carbohydrate ligands[40].

In addition to mono- and linear oligosaccharide structures, a diversity of complex oligosaccharides have been identified as high-affinity ligands for NKR-P1 [41]. These ligands contain oligosaccharide sequences of the blood group family, the ganglio family, and glycosaminoglycans[41]. Moreover, it has been shown that interactions of such oligosaccharides on tumor cell surface with NKR-P1 on the surface of NK cells are crutial both for tumor cell recognition, and for natural killing[41]. NK resistant tumor cells are rendered susceptible by preincubation with liposomes containing NKR-P1 ligands suggesting a possibility for purging of tumor or virally infected cells in vivo[41]. Cancer immunomodulation by NKR-P1 carbohydrate ligands has been indeed shown to result in the reduction of tumor incidence similar to that caused by chemotherapy (5-fluorouracil control)[42]. However, we met considerable problems when developing the above approach caused by limited availability, poor stability and considerable costs of the complex oligosaccharide ligands. In addition, some of the original findings[24,41] have been later questioned[43,44], and it took some time to resolve the controversy[45].

For all these reasons, we turned in the meantime our attention to a new generation of high-affinity ligands for NKR-P1, namely to synthetic ligand mimetics based on simple and stable monosaccharide ligands for NKR-P1. These compounds are based on polyamidoamine or polylysine backbones to which β-*N*-acetyl-D-glucosamine is linked via a stable *N*- or *S*-glycosidic linkage[46]. In the initial testing, these carbohydrate-coated dendrimers proved to be efficient ligands for NKR-P1 receptor in vitro[46]. Moreover, these compounds are very efficient as therapeutic agents in experimental cancer without any need to combine them with liposomes[47]. Advantages in survival of experimental animals and reduction of tumor growth were obtained in a dose dependent manner after intraperitoneal or intracardial application of carbohydrate dendrimer solutions. Unlike other carbohydrates used in the tumor vaccination programs, which lead to a massive production of antibodies that might eventually destroy the tumors, the carbohydrate dendrimers were shown to engage the cellular branch of the immune system (both innate and acquired). This engagement may turn crutial for the destruction of the tumors that have developed into a critical size[48].

The pattern of carbohydrate binding by human CD69, the second NK cell receptor investigated in our laboratory, turned out to be completely different from what we have seen for the rat NKR-P1 receptors. During our detailed biochemical studies we have obtained evidence that binding of calcium to CD69 results in a significant space shift in the adjacent Thr^{107} and Lys^{172} residues. Such shift is essential for the formation of the high-affinity binding site for GlcNAc (Fig. 8). Direct binding studies employing equilibrium dialysis and other techniques revealed the existence of three binding sites for GlcNAc in the NKD of CD69 with unequal binding affinities[31]. Detailed analyses of the binding data recorded for wild-type and several mutant proteins together with molecular modeling and molecular docking allowed to define precisely the position of all three GlcNAc-binding sites[31]. Binding sites for GalNAc are partially overlapping with the GlcNAc-binding sites, but ManNAc is not recognized by CD69. This peculiar positioning of the monosaccharide-binding sites in CD69 led to a prediction that complex, high-affinity ligands for the receptor would be among the branched oligosaccharides as opposed to the linear structures recognized by NKR-P1 receptors. Indeed, screening of highly branched GlcNAc-terminated oligosaccharides obtained from egg white glycoproteins allowed to identify high-affinity ligands of this type among the pentaantennary ovomucoid-type oligosaccharides[49]. Pentaantennary oligosaccharide structure of this type docks into CD69 primarily through the three terminal GlcNAc residues, but extensive contacts occur over much larger surface of the oligosaccharide beyond the three terminal sugar residues docked into the monosaccharide-binding sites. The high affinity of this interaction is also supported by direct interaction of both the oligosaccharide and the receptor with calcium that has been lifted up from its usual position by the

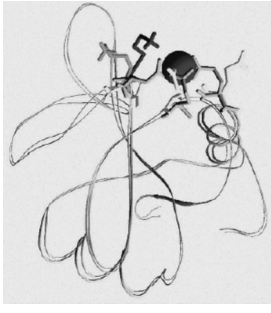

FIG. 8
Structural changes upon binding of calcium to CD69 [34]

high-affinity ligand. Although the physiological relevance of binding of these ligands for biology of CD69 remains to be established, it is worth mentioning that complex ovomucoid-type carbohydrates are present on the surface of malignantly transformed cells in the form of hyperbranched N-linked oligosaccharides. These results may thus be relevant to the immunobiological role of CD69 especially in view of the unknown physiological ligand for CD69. Our preliminary findings indicate the ability of ovomucoid and its oligosaccharides to induce apoptosis in $CD69^+$ cells[49].

In the concluding part of this section it may be worth mentioning the ligand identification experiments performed recently in our laboratory with the other members of the NK cell receptor family. NKG2D, which is a very important activation receptor of NK cells directly involved in the recognition of malignant, virally infected and even stressed cells[50,51], was shown to bind to GlcNAc, GalNAc and also to Man when presented in the form of neoglycoproteins (Fig. 9, upper right panel). DCL-1 receptor, which belongs to the new, rapidly growing family of C-type receptors expressed on dendritic cells[52,53], was shown to recognize β-linked glucose, β-linked galactose, and α-linked fucose (Fig. 9, lower right panel). Considering the growing number of molecules in the NK receptor family, and their overlapping carbohydrate specificities and diverse surface expression on various leukocyte, it may appear very difficult to design carbohydrate-based structures for specific targeting to the individual receptors. While this is certainly true for the level of monosaccharides, one may still attain a high degree of selectiv-

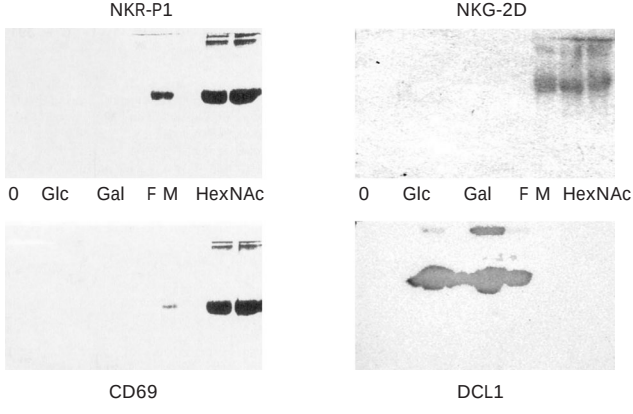

FIG. 9
Carbohydrate-binding fingerprint for four model NK cell receptors. The technique of neoglycoprotein overlays has been employed using BSA derivatives of the indicated monosaccharides that are available commercially

ity using well selected complex oligosaccharides. Examples of four such oligosaccharide ligands that are rather selective for the four NK cell receptors shown in Fig. 9 are given in Fig. 10. It should be evident from the data shown in Fig. 10 and from the discussion in the previous text that going from the monosaccharide to the oligosaccharide level both specific and selective carbohydrate ligands may be found for the limited set of receptors examined so far. These specific ligands are chitotetrose for NKR-P1A, branched ovomucoid-type *N*-glycans for CD69, high-mannose *N*-glycans for NKG2D, and β-glucan from barley for DCL-1. Further research will have to reveal the extent of the overlapping carbohydrate specificity pertinent to the increase in the number of examined NK cell receptors. Also, it will be interesting to see if it is still possible to identify specific ligands among the complex glycoconjugates.

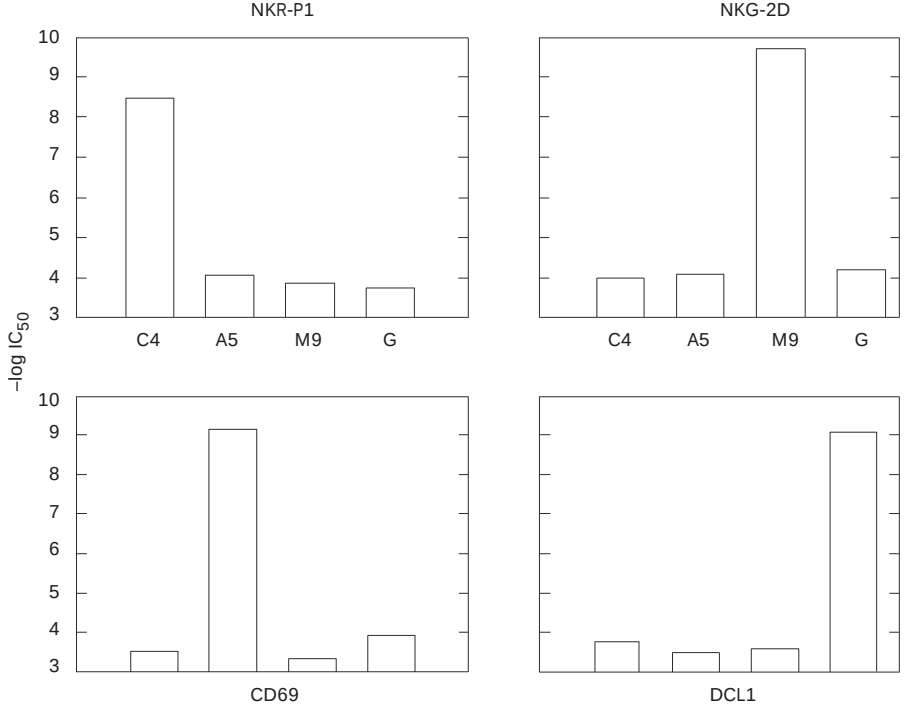

FIG. 10
Oligosaccharide ligands displaying selective binding to the four NK cell receptors shown in Fig. 9. These oligosaccharide ligands include chitotetrose (C4), pentaantennary oligosaccharide A5, high-mannose oligosaccharide M9, and barley β-glucan. The affinity of the individual receptors for these oligosaccharide ligands is expressed as $-\log IC_{50}$

5. REMODELING OF THE BINDING SURFACE TO ACCOMMODATE MHC CLASS I PROTEIN LIGANDS

As already mentioned in the evolutionary chapter, multiple sequence alignments would predict that the ligand-binding surface in NKDs of NK cell receptors is evolutionarily distant from the corresponding classic C-type animal lectins, and has been remodeled to accommodate other types of ligands in addition to calcium and carbohydrates. Initially it was not possible to predict which chemical structures would be a possible candidate for the additional ligands of NK cell receptors. However, a number of functional (immunological), binding, and, eventually, structural studies have been performed in the recent decade that have dramatically clarified this issue. Immunologists studying the Ly49 cell surface receptors began to uncover the fine binding specificities of these receptors towards cells expressing varying levels of MHC class I antigens in the 1980's and early 1990's [54]. With the rapid progress in the genetics of rodent and human NK cell gene complex, many Ly49 receptors were cloned and sequenced, which opened the way for the binding experiments with defined molecules. These were first done using cell lines transfected with individual Ly49 molecules in cellular assays, and later defined on recombinantly prepared proteins in biochemical assays. Early functional studies and successful recombinant expression of the relevant molecules found their reflection also in the structural studies. The milestone in this area was the solution of the structure of CD94 [55], and cocrystallization of an important mouse inhibitory receptor Ly49A with mouse H-2^k (literature[56]), which provided initial structural paradigms for more detailed examinations of NK cell receptor–MHC class I interactions. It was immediately apparent from these early studies that the ligand-binding surface in NKDs was subjected to a dramatic structural rearrangement. As an example, one of the α helices found in many structurally defined CTLDs was replaced by β-sheets in CD94 in order to promote interactions with MHC class I glycoproteins (see Fig. 4). Similarly, diversity in the ligand-binding loops of Ly49A has increased the ligand-binding area to an extent that makes it easy to promote interactions with MHC class I glycoproteins. These early studies have since provided an important conceptual framework for the discussion of a large amount of experimental data that have been since obtained on NK cell receptor–MHC class I interactions.

In the mouse, the only known NK cell receptors for the classic MHC class I molecules belong to the Ly49 receptor family[57]. Seven well defined Ly49 family members contain inhibitory motifs in their cytoplasmic domains, namely Ly49A, -B, -C, -E, -F, -G2 and -I and have been described to be ex-

pressed at least at the mRNA level in NK cells from C57BL/6 (B6) mice[54]. Based on functional studies, Ly49A was initially thought to be specific for D^d, but not K^d, L^d, K^b or D^b (literature[54]). Direct binding of Ly49A to D^d was demonstrated by cell adhesion assays[58] and plate-binding assays[59]. Because $Ly49G2^+$ NK cells lysed $H\text{-}2^d$ cells more efficiently in the presence of monoclonal antibodies against D^d or L^d, this receptor was assigned a specificity for D^d or L^d (literature[60]). However, direct evidence for a physical interaction between Ly49G2 and D^d has not been reported. Cell adhesion experiments with Ly49C and Ly49I-transfected COS-7 cells and MHC disparate tumor cell lines demonstrated that Ly49C binds to $H\text{-}2^b$, $H\text{-}2^d$ and $H\text{-}2^k$, but not $H\text{-}2^s$ gene products, while Ly49I was not found to bind any of these antigens[61,62]. Until recently, little has been known about the expression and MHC reactivities of any of the other Ly49 receptors with predicted inhibitory function (i.e. Ly49B, -E and -F). Ly49D exhibits activating rather than inhibitory function in response to target cells expressing the D^d class I molecule[63,64] but no binding data have been reported.

Available evidence indicates that Ly49A interacts with the $\alpha 2$ domain of D^d (literature[58,65]), with possible contribution from the $\alpha 1$ domain[66]. The functional binding of Ly49A to D^d showed no peptide specificity since a variety of peptides that stabilize D^d expression on target cells protects these from lysis by $Ly49A^+$ NK cells[67,68]. Ly49 receptors are expressed on partially overlapping subpopulations of NK cells[69]. The missing self-hypothesis proposes that NK cells should be inhibited by the few self-class I molecules inherited by each individual. It is believed that education processes ensure that many or all functional NK cells express self-class I specific receptors[69,70]. In order to understand the molecular interaction accompanying these complicated immunological processes, two binding assays and a functional assay were employed to examine the specificities of the entire panel of inhibitory Ly49 receptors for eight MHC haplotypes[71]. Different Ly49 receptors exhibited diverse binding properties, and the degree of class I binding has always been related to the extent of functional inhibition. The binding studies using MHC class I tetramers demonstrated that neither glycosylation nor coreceptors were necessary for class I binding. Recognition of Ly49 in the latter study was found to be peptide-specific. Seven of the nine Ly49 genes expressed in C57BL/6 mice contain an immunoreceptor tyrosine inhibitory motif (ITIM) in their cytoplasmic domain which is responsible for the inhibitory function. However, Ly49D and Ly49H are different since they lack the ITIM, and function as activating receptors through their noncovalent association with the membrane adaptor DAP-12 [72].

The CD94/NKG2A receptor that is conserved in both rodents and primates is an example of a disulfide-linked homodimer composed of subunit coded by separate *CD94* and *NKG2A* genes[73]. The CD94/NKG2A heterodimer is typically expressed on about half of NK cells, and on a subset of memory $CD8^+$ T cells. Whereas CD94 has a short cytoplasmic tail lacking signaling function, NKG2A contains two ITIMs, which can, upon tyrosine phosphorylation, recruit SHP-1 and SHP-2 phosphatases responsible for the propagation of the inhibitory signals[74,75]. This heterodimeric receptor recognizes human HLA-E and the homologous mouse $Qa1^b$ molecule. The peptide-binding groove of HLA-E and $Qa1^b$ is often occupied by 9 amino acid peptides derived from the signal peptide of other MHC class I molecules such as H-2D, H-2K, HLA-A, HLA-B, HLA-C and HLA-G [76,77]. In the absence of these peptides, HLA-E and $Qa1^b$ are retained in the cytoplasm of the host cells and degraded. This provides NK cells or T cells expressing CD94/NKG2A with an efficient mechanism of monitoring the expression of MHC class I on a variety of tissues based on the model shown in Fig. 1. The related CD94/NKG2C heterodimeric complex also binds HLA-E/$Qa1^b$ but NKG2C lacks an ITIM and associates with DAP-12 to form an activating receptor complex[72]. As has already been mentioned, the inhibitory NK cell receptors perform their function through association with SHP-1 and SHP-2 protein tyrosine phosphatases. As yet, however, there is little definitive data about the downstream substrates for these enzymes although SLP-76, LAT, CD3ξ and FcRIγ have all been suggested as possible targets[78,79]. Inhibitory NK cell receptors have been implicated in rejection of bone marrow allografts, in the elimination of tumors lacking MHC class I, and in protection against viral infections[72].

6. OTHER TYPES OF LIGANDS

While the inhibitory NK cell receptors are certainly vital for the protection against uncontrolled killing of healthy cells, much attention has been recently paid to the activation receptors that turn the natural killer cells on. It appears from a large amount of experimental data that NK cells do not possess a single predominant activation receptor ("*receptor magnificus*") similar to B-cell or T-cell receptors. Rather, NK cells seem to use a variety of different, nonrearranging receptors to initiate cytolytic activity and cytokine production[80,81]. These receptors that are nonstochastically coexpressed on NK cell surface[82] include certain immunoglobulin superfamily molecules not yet mentioned here, such as, CD2 [83], CD16 [84], CD44 [85], 2B4 [86], DNAM-1 [87], NKp44 [88], NKp46 [89], and NKp30 [90]. Moreover, there are C-type

lectin family molecules that have already been discussed in the above paragraphs, such as NKR-P1 [91,92], CD69 [93], and CD94/NKG2C [94]. Furthermore, NKG2D receptor has recently attracted considerable attention of immmunologists as one of the most important activating receptor on NK cells[95] with a direct link to killing of tumor, virally infected or even stressed and otherwise damaged cells[50,96]. NKG2D is expressed on NK cells, $CD8^+$ αβ T cells, γδ T cells and macrophages making it one of the most widely distributed NK cell receptors described so far[97]. Other members of the NKG2 family (A, C, E and F) described before form obligate heterodimers with CD94, are highly homologous to each other (approximately 90% identical), and are specific for the non-classic MHC class I protein HLA-E (see above). On the other hand, NKG2D displays only limited sequence similarity to other NKG2 molecules (20–30% identities), have not been demonstrated to interact with the classic MHC class I, and form heterodimers[98,99]. The ability of NKG2D to trigger costimulatory signals in $CD8^+$ T cells and cytotoxicity in NK cells is due to its association with two different intracellular adaptors, DAP10 and DAP12 [100,101]. Ligands for human NKG2D include three closely related proteins, MICA, MICB and ULBP (cytomegalovirus UL 16-binding protein)[102–105]. They are distant MHC class I homologs that do not function in conventional mode of presentation of the antigenic peptides. Also, unlike the widely and constitutively expressed classic MHC class I proteins, MICA and MICB are induced on the cell surface in response to cellular stress or transformation of the intestinal epithelium into epithelium-derived tumors[106]. While MICA and MICB are quite similar to each other (84% identical), they have diverged significantly from the classic MHC class I family as a whole, with domain-by-domain identities of only 28–35%. The evolutionary divergence is reflected in important functional differences in that MICA and MICB do not require the bound peptide for either surface expression or NKG2D recognition. These proteins are also expressed on the cell surface independently of β_2-microglobulin. ULBPs are homologous to the $\alpha_1\alpha_2$ peptide-binding platform domains of MHC class I, but lack the α_3 domains, and are bound to the plasma membrane by a glycerolphosphatidylinositol anchor. The functional significance of the interaction of human NKG2D with ULBP1 remains to be determined. It has been shown that these viral proteins may inhibit the physiological interaction of NKG2D with MHC class I – like antigens, and thus potentially represent a viral strategy to eliminate the immune recognition and destruction. Indeed, the recent report has provided evidence that the human cytomegalovirus protein UL16 can cause intracellular sequestration of NKG2D ligands, thus protecting the infected cell against natural killer cell cytotoxicity[107]. CTLD

of murine NKG2D protein is 69% identical to that of human NKG2D suggesting a possibility that similar ligands might be recognized by the mouse orthologue. However, rodents lack any known homologs of MICA and MICB. Nevertheless, ligands for murine NKG2D have been identified among RAE-1 (retinoic acid early inducible) family of proteins and H60 [108–110]. Like the ULBPs, REA-1 and H60 are homologous to the platform domains of MHC class I proteins, lack α_3 domains, and are anchored by glycosylphosphatidylinositol. Isoforms of RAE-1 (α, β, γ, and δ) are highly expressed during embryonic development, but are rare in normal adult tissues[109]. It has been recently shown that tumors expressing REA-1 molecules can be recognized by NK cells and rejected[111]. Like human NKG2D–MIC stimulation of NK cells, REA-1 mediated rejection is interesting since it can override inhibitory signals from the expression of self-MHC class I on the tumor cells. H60 was originally identified as an immunodominant minor histocompatibility antigen[112]. Though differentially expressed on imbred mouse strains, H60 transcripts were found at low level in embryonic tissues but at higher levels on macrophages and dendritic cells in the spleen[113].

Most interactions of NK cell receptors and T-cell receptors with their ligands have dissociation constant in the 10^{-5} mol l^{-1} range[114] (Table I). However, interactions of human and murine NKG2D with their ligands are one to two magnitudes stronger (Table I). Kinetic analysis of the interaction of human NKG2D with MICA yielded a dissociation rate constant (k_{off}) at 37 °C of 0.04 s^{-1}, indicating higher stability than observed for interactions of other NK cell and T-cell receptors[98]. The association rate constant (k_{on}) in the range 4–7 × 10^4 l mol^{-1} s^{-1}, which was measured for NKG2D interac-

TABLE I
Immunoreceptor affinities (modified according to[114])

Receptor	Ligand	K_D, μmol l^{-1}
T-cell receptor	MHC class I	1–90
CD8	MHC class I	65–200
NKG2A-CD94	HLA-E	11
KIR	MHC class I	10
Human NKG2D	MICA	0.3
Murine NKG2D	H60	0.02
Murine NKG2D	RAE-1α, β, γ, δ	0.3–0.7

tions, is relatively slow when compared with other immune receptors that typically have $k_{on} > 10^5$ l mol^{-1} s^{-1} (literature[115]). These analyses also showed that both association and dissociation rate constants are somewhat temperature-dependent indicating that activation energy barriers impede association and dissociation. Binding studies show that RAE-1 and H60 compete directly for occupancy of the murine NKG2D demonstrating the overlapping binding sites[116]. This interaction of murine NKG2D with H60 is more temperature-dependent, and makes greater use of electrostatic interactions than NKG2D–RAE-1 interaction[116]. Entropy changes during binding suggest that dominant energy gain in NKG2D–H60 interaction comes from the stabilization of flexible protein loops at the binding interface, while in the case of NKG2D–RAE-1 interaction, displacement of the bound water is dominant[116]. The binding analysis of the interactions of murine NKG2D with H60 and RAE-1, and gel permeation chromatography of the complex of human NKG2D with MICA indicate that the stoichiometry of binding in the NKG2D complexes is 2:1, meaning that one NKG2D homodimer binds a single monomeric ligand[99] (MICA, REA-1 or H60). This binding mode is somewhat unprecedented since the simplest complex one would expect is the 2:2 complex, in which two molecules of the ligand would occupy the two binding sites in the dimeric NKG2D protein. The 2:1 complexes observed here can be formed when the symetric receptor-binding sites are positioned in a way that binding of ligand at one site would block binding at the second (a documented example of such an interaction includes Ly49 complex with H-2D^d)[56], or when the receptor-binding sites have evolved to recognize two distinct sites on the surface of the ligand (examples for the latter type of interactions being binding of CD8 to HLA[117], or the human growth hormone receptor–ligand complex[118]).

Crystal structures of MICA[119] and RAE-1[120] revealed very distorted MHC class I platform domains. The platform domain in the classic MHC class I proteins comprises two long, roughly parallel, α helices arranged on a platform of eight-stranded β-sheet. The helices define the peptide binding groove in MHC class I homologs that bind the antigenic peptides. The novel structural feature of MICA includes a ten-residue segment corresponding to the central section of the α_2 domain helix in the MHC class I which is disordered to form a flexible loop. Moreover, the platform and α_3 domains in MICA are joined through a flexible linker, allowing considerable interdomain flexibility, a feature unique to MICA among MHC class I homologs. The structure of RAE-1 is distorted from other MHC homologs in the arrangement of helical elements and displayed noncanonical disulfide

bonds, one of which links the α_1 and α_2 domains. The loss of any remnant of the peptide-binding groove in RAE-1 is facilitated by the close approach of the groove-defining helices through a hydrophobic, leucine-rich interface. However, other MHC class I homologs also close off the peptide-binding groove, mostly through salt bridges. Interestingly, MICA actually retains a small pocket in the center of the platform domain, but due to its size and polar character it is unlikely to bind any peptide or other small molecule as a specific ligand.

Recent crystal structures of the MICA–human NKG2D and RAE-1–murine NKG2D complexes[98,119] revealed that NKG2D homodimers bound to both MICA and RAE-1 monomers through an interaction analogous to αβ T-cell receptor–MHC class I protein complexes, with the NKG2D homodimer diagonally overlaid on the surface of the ligand platform. This finding of very similar structural arrangement is surprising in view of the fact that T-cell receptors are constructed from immunoglobulin-like domains while NKG2D is assembled from NKD domains. Moreover, there are significant structural differences between the platform domains of MHC class I proteins and their MICA and RAE-1 homologs. From the structural measurements it became obvious that the interaction between human NKG2D and MICA is more extensive and involves greater shape complementarity than most interactions between T-cell receptors or NKD domains and MHC class I (Table II). On the other hand, the complex between murine NKG2D and RAE-1 displays interface values more typical of the immune receptor complexes (Table II). Nevertheless, the shape complementarity in both complexes is sufficient to completely exclude solvent from the interface, and no solvent-mediated contacts are seen in either crystal structure. It is interesting to emphasize

TABLE II
Structural parameters of NK cell complexes (modified according to[114])

Complex	Buried surface area, Å^2	Surface complementarity
Typical protein/protein	1600	0.64–0.68
T-cell receptor/MHC	1700–1900	0.43–0.70
KIR/MHC class I	1485–1540	0.69–0.71
Ly49A/H-2D^d(site 1)	990	0.78
Ly49/H-2D^d(site 2)	3350	0.54
Human NKG2D/MICA	2180	0.72
Murine NKG2D/RAE-1	1700	0.63

that while still retaining a very high affinity for interactions with ligands, the binding site in NKG2D has a remarkable plasticity which allows it to tolerate numerous, dramatic substitutions and deletions of ligand contact residues. For instance, murine NKG2D binds to all of the RAE-1 isoforms over an approximately two-fold range of affinity (cf. Table I). While some of the amino acid substitutions among these isoforms are assumed to be quite conservative, some of them are quite dramatic and large changes in the binding affinities could be predicted. While the nature of this phenomenon is not yet completely understood, it can have profound biological effects in the process of "fine tuning" of the immune receptors.

In the preceding sections we have discussed instances in which peptides, such as antigenic peptides that are bound to the antigen-presenting groove of MHC class I, influence the interaction of C-type lectin-like NK cell receptors with their specific ligands. The influence of MHC class I-bound peptide on recognition by inhibitory receptors of NK cells had been previously reported for the immunoglobulin family members, namely for the KIR3DL and KIR2DL receptors specific for HLA-B and HLA-C, respectively[120,121]. Although many different peptides are compatible with recognition by KIR receptors, certain side chains at positions 7 and 8 of the nonamer peptide interfere with binding. However, crystal structures of the two KIR complexes mentioned above revealed why these receptors bind only in the presence of peptides: direct contacts are made between KIR and the peptide backbone, and certain peptide side chains cannot be accommodated in the tight space[122,123]. Recently, a similar mechanism has been revealed for the inhibiton of the interaction of NKG2A/CD94 heterodimer with HLA-E [124]. Since it is known that the amino acid sequence of HLA-E bound peptide would affect the affinity of this interaction, a search for the specific peptide inhibitor has become very attractive. In the new study, a peptide from the signal sequence of stress protein hsp60 was shown to load onto HLA-E and to compete effectively with the MHC class I-derived peptide[124]. This competitive peptide replacement would be feasible under physiological conditions, and may represent an important mechanism for the viral modulation of the immune response.

C-Type lectin-like domains found in receptors expressed on leukocyte subsets other than NK cells have recently attracted considerable attention. These receptors represent a rich source of examples for the diversity of ligands that may be recognized by these protein modules under different physiological conditions. One interesting example worth mentioning here is CD23 antigen, which is type II membrane protein containing the C-terminal extracellular CTLD. This molecule is a low-affinity receptor for im-

munoglobulin E (IgE) important in allergies and immunity against parasites[125]. Low affinity of interaction of this receptor with IgE together with abundant glycosylation found in this particular immunoglobulin class has been indicative of a possibility of lectin-type interaction. However, no evidence could be obtained for such an interaction during the detailed molecular studies, although the soluble CD23 can interact with another important activation receptor of B cells, CD21, through carbohydrates[126]. C-Type lectin receptors on dendritic cells have already been mentioned[52,53]. These molecules recognize β-glucan and other microbial polysaccharides, and thus represent an important receptor system for these compounds that has been anticipated for many years yet difficult to find[53]. An interesting array of ligands has been identified for C-type lectin named LOX-1. This molecule is expressed on endothelial cells, where it can bind oxidized low-density lipoprotein[127], apoptotic bodies[128], support adhesion to fibronectin[129], and take up the advanced glycation end products[130], or even products of Gram-positive bacteria[131]. This broad reactivity would define LOX-1 as an important scavenger receptor distinct from class A and B receptors[127]. Amino acid residues responsible for binding of ligands in LOX-1 have been identified[132]. The peptide mimetics competing for the binding of natural ligands have been also developed, and proved attractive in the targeting of genes into epithelial cells[133].

To conclude this section, instances are discussed in which the peptide itself is becoming an important ligand for NK cell receptors. Although a peptide-binding sites within the NKDs have not been mapped extensively so far, we have recently found a specific interaction of the peptides derived from mycobacterial heat shock proteins, such as hsp65 from *Mycobacterium bovis* with CD69 [134]. Interestingly, homologous peptides from human hsp60 or hsp70 proteins are not recognized by this receptor[134]. The interaction has high affinity with K_d in the 10^{-8} mol l^{-1} range, so that the specific peptide derived from the mycobacterial pathogen (but not the corresponding human peptide) can displace the high-affinity oligosaccharide ligand (K_d of the order 10^{-7} mol l^{-1}) from its binding site[134]. The immunological relevance of this phenomenon is not completely understood although it may certainly be an interesting example of the modulation of immune response by peptides of microbial origin.

7. CONCLUSIONS

Although we have discussed interesting recent examples of the remarkable variability of NKD surface to engage a wide range of ligands, it remains un-

clear how exactly the NK cell receptors may tolerate such a plasticity of the ligand-binding sites while still retaining considerable specificity and high affinity. Obviously, despite a large amount of experimental work, we are seeing only the tip of the iceberg, and our understanding of the above phenomenon is far from complete. Although further structural work based on crystallographic, binding and mutagenesis studies will be required to uncover the rules of molecular recognition for these receptors systems, the functional role of this ligand polymorphism remains even more interesting. One explanation would be that by varying the affinity (and, therefore, the half-life) in these receptor–ligand interactions, signals with a large range of strengths may be created. This would then allow a greater flexibility in the decision of the effector NK cells to kill or not to kill the targets, depending upon the activation threshold set for the individual constellations of stimulatory and inhibitory receptors.

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